

THE PREPARATION OF 2,3 (³H)-GA₂₉ AND ITS METABOLISM BY ETIOLATED SEEDLINGS AND GERMINATING SEEDS OF DWARF *PISUM SATIVUM* (METEOR)

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ABSTRACT

(³H)-GA₂₉ was prepared biosynthetically from (³H)-GA₂₀ by germinating pea seeds. (³H)-GA₂₉ applied to dark-grown seedlings and germinating seeds of dwarf *Pisum* was converted into polar, acidic products, but analysis of these products by mass spectrometry was rendered impossible by low levels of metabolites, and a lack of unlabelled GA₂₉ with which to increase metabolite pool-sizes. The results are discussed in relation to recent attempts to investigate the pathways of gibberellin biosynthesis in higher plants, and to draw attention to some of the problems associated with these investigations.

UITTREKSEL

DIE BEREIDING VAN 2,3 (³H)-GA₂₉ EN DIE METABOLISME DAARVAN DEUR GEËTIOLEERDE SAAILINGE EN ONTKIEMENDE SADE VAN DWERG *PISUM SATIVUM* (METEOR)

(³H)-GA₂₉ is biosinteties van (³H)-GA₂₀ voorberei deur eertjiesade te ontkiem. (³H)-GA₂₉ toegedien aan in die donker gekweekte saailinge en ontkiemende sade van dwerg *Pisum* is verander tot polare suurvormendeprodukte maar ontleding van die produkte deur massa spektrometrie was onmoontlik weens die lae vlakke van metaboliet en die afwesigheid van ongemerkte GA₂₉ om die metabolietpoel te vergroot. Die resultate word bespreek in die lig van die jongste pogings om die gibberellin biosintese roete in hoër plante na te speur en om die aandag op sekere probleme verbonde aan hierdie navorsing, te verlig.

INTRODUCTION

Studies have recently been conducted to elucidate the pathway(s) of gibberellin (GA) biosynthesis in intact seedlings of dwarf *Pisum* (Durley, Railton and Pharis, 1973; Durley, Railton, and Pharis, 1974, 1975; Railton, Durley and Pharis, 1974a, 1975a; Railton, Murofushi, Durley and Pharis, 1974b). Preliminary studies were of a somewhat exploratory nature and utilised some labelled precursors and intermediates which were not known to be native pea GAs. Thus, 1,2 (³H)-GA₅ and 1,2 (³H)-GA₁, two GAs believed on circumstantial evidence, to be native to dwarf pea (Kende and Lang, 1964), were converted respectively, to GA₃ (Durley *et al.*, 1973) and the diol, GA₈ (Railton, Durley and Pharis, unpublished data). Similarly, 16, 17 (³H)-GA₁₄, an early intermediate in the GA biosynthetic pathway in the fungus, *Gibberella fujikuroi*

(Cross *et al.*, 1968), was metabolised by etiolated dwarf pea seedlings to GA₁₈, GA₃₈, GA₂₃, GA₁, GA₈ (Durley *et al.*, 1974, 1975) and a new GA, GA₁₄-hydrate (Durley *et al.*, 1975) which has recently been isolated from *G. fujikuroi* and allocated the "A" number, GA₄₂ (Bearder and MacMillan, 1973).

Concurrent with metabolism studies, attempts were made to investigate the GA content of the pea seedling. It had previously been assumed that pea seedlings contained only 2 GA-like substances, one similar to GA₃ and/or GA₁, and the other similar to GA₅ and/or GA₂₀ (Kende and Lang, 1964; Jones and Lang, 1968). A reinvestigation of this work using different analytical techniques, indicated the presence of at least 6 GA-like substances in pea seedlings, one of which was similar to GA₉ (Railton and Reid, 1974; Railton, Durley and Pharis, unpublished data, 1972). In parallel studies, MacMillan and co-workers have recently investigated the GA content of immature seed of dwarf pea and have characterised, by combined gas chromatography-mass spectrometry (GC-MS), GA₉, GA₁₇, GA₂₀, GA₂₉, GA₃₈, and 13, hydroxy-GA₁₅, as native pea GAs (Frydman and MacMillan, 1973; Frydman *et al.*, 1974). The GA, 13, hydroxy-GA₁₅ was previously synthesised from GA₁₉ by borohydride reduction (Fukui *et al.*, 1972) and has now been allocated the "A" number GA₄₄ (Frydman *et al.*, 1974). Application of 16, 17 (³H)-GA₉ to intact dwarf pea seedlings, showed it was converted into GA₂₀ (I), GA₂₉ (II), the hitherto unknown GA, 2,3 dihydro-GA₃₁, and the hydrate of GA₉, GA₁₀ (Railton *et al.*, 1974a, 1975a). When 2,3 (³H)-GA₂₀ was fed to dwarf pea, it was hydroxylated, specifically, at C-2 to produce GA₂₉ in high yield (Railton, Murofushi, Durley and Pharis, 1974b).

The demonstration that GA₉ is converted *via* GA₂₀ to GA₂₉, indicates that these 3 GAs form part of a biosynthetic sequence in the pea plant. Furthermore, the presence of GA₁₇, GA₃₈ and GA₄₄ suggests the existence of more than one GA biosynthetic pathway in dwarf *Pisum*. In order to investigate further the biosynthesis of GAs in *Pisum*, it is necessary therefore, to produce in labelled form, other native pea GAs. Difficulties in total chemical synthesis and the unavailability of many GAs in reasonable quantities has limited the production of radioactively labelled GAs. In the present paper, the biosynthetic preparation of 2,3 (³H)-GA₂₉ of high radionuclidic purity is reported and preliminary attempts described to study its metabolism in dwarf *Pisum sativum*.

MATERIAL AND METHODS

Synthesis of 2,3 (³H)-GA₂₀. This was prepared by the method of Murofushi *et al.*, (1974). Gibberellin A₅ was converted into its methyl ester with ethereal diazomethane and then treated with *meta* chloroperbenzoic acid to afford the epoxide of GA₅ methyl ester. Reduction of this epoxide with tritium yielded 2,3 (³H)-GA₂₀ methyl ester epoxide. Cleavage of the epoxide ring was achieved with

iodine and zinc dust and the ester hydrolysed with 0.2N sodium hydroxide to afford 2,3 (^3H)-GA₂₀, sp. act 2.0 Ci/mM.

Preparation of 2,3 (^3H)-GA₂₉. Dry, mature seeds of dwarf *Pisum sativum* L. (Meteor) were imbibed in distilled water containing 10 μ Ci (0.6 μ g) 2,3 (^3H)-GA₂₀ under continuous illumination at 22°C. A total of 15 seeds were used and following radical emergence, were ground in a mortar with acid-washed sand and chilled 80% methanol. All methanol was removed *in vacuo* at 35°C, the remaining aqueous phase adjusted to pH 9.0 with an equal volume of 0.5M

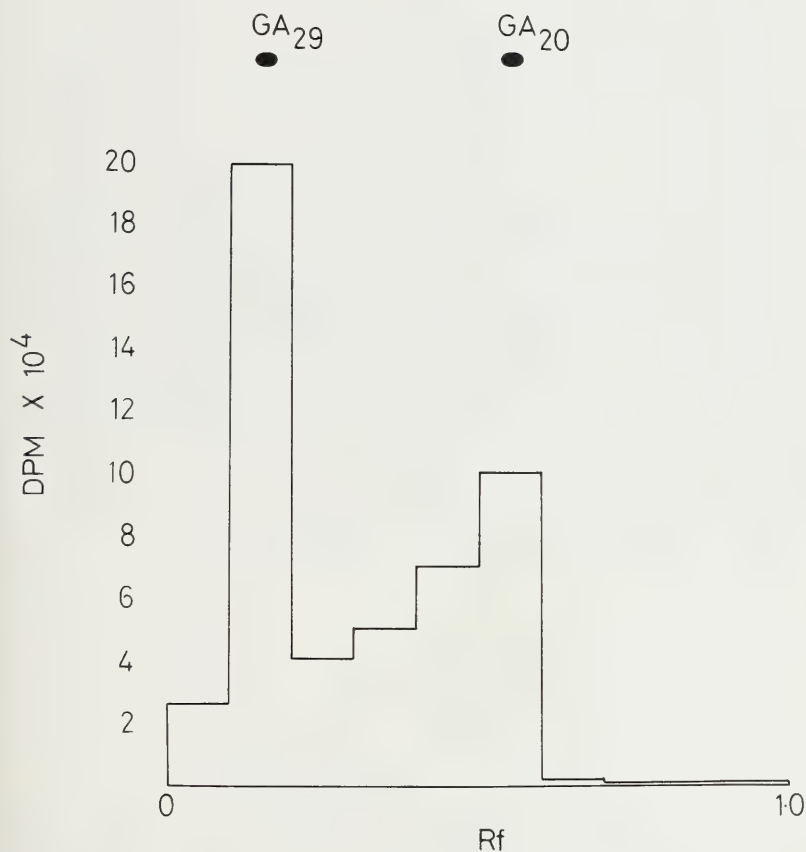


FIG. 1
Preparation of (^3H)-GH₂₉ and its metabolism in dwarf *Pisum*.

phosphate buffer (pH9.0) and partitioned against diethyl ether to remove basic/neutral impurities. The aqueous phase was then adjusted to pH 3.0 with IN HCl and partitioned 6X against ethyl acetate. The ethyl acetate fraction was reduced to dryness *in vacuo* and the residue chromatographed on preparative TLC using silica gel H and the solvent system ethyl acetate, chloroform, formic acid (50:50:1, v/v). A single zone of radioactivity (Rf 0.1–0.2) corresponding to a marker of authentic GA₂₉ (Figure 1) was eluted with water-saturated ethyl acetate and then reduced to dryness.

PURIFICATION

(i) *PVP column chromatography.* The eluate from the TLC plates was purified further by column chromatography using insoluble poly-N-vinyl pyrrolidone (PVP) eluted with 0.1 M phosphate buffer, pH 8.0 (Glenn *et al.*, 1971).

(ii) *Silica gel partition column chromatography.* GA₂₉ recovered from the PVP column was finally purified by high efficiency silica gel partition column chromatography using a modification of the method of Powell and Tautvydas (1967). This was carried out as follows: silica gel (Mallinkrodt CC4, for column chromatography) was dried to constant weight in an oven and then slurried with a calculated amount of 0.4M formic acid until the gel had the consistency of moist sand. After leaving to equilibrate for 4 days, the liquid coated support phase was slurried in formic acid-saturated n-hexane and degassed under vacuum. The column was packed in n-hexane in a nitrogen enriched atmosphere to minimise air uptake by the solvents during packing. Failure to do this results in partial "stripping" of the liquid stationary phase during chromatography

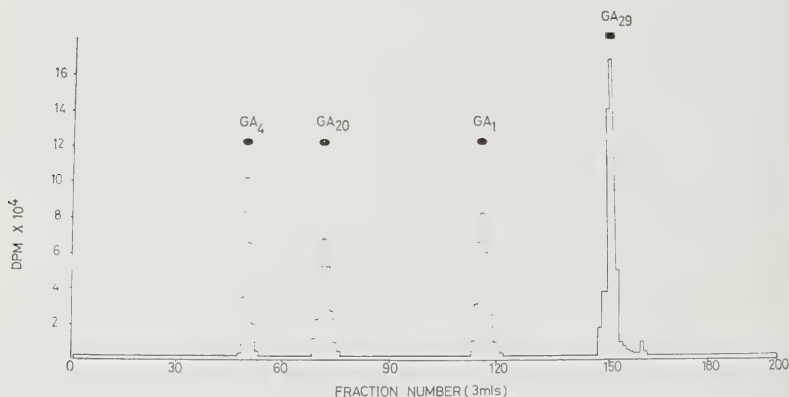


FIG. 2
Preparation of (³H)-GA₂₉ and its metabolism in dwarf *Pisum*.

with marked reductions in column efficiency (Kirkland, 1971). The partially purified radioactive GA₂₉ sample was introduced on to the top of the column in 100 μL of ethyl acetate and the column eluted with *n*-hexane containing increasing percentages of ethyl acetate. GA₂₉ eluted as a discrete peak (Figure 2) which was collected, reduced to dryness and an aliquot taken for analysis by gas liquid-radiochromatography (GLRC).

(iii) *Gas liquid-radiochromatography*. GLRC analysis was carried out in a similar manner to that described previously (Durley *et al.*, 1973; Durley *et al.*, 1974, 1975, Railton *et al.*, 1974a). 2,3 (^3H)-GA₂₉ methyl ester, trimethylsilyl ether chromatographed as a single peak on GLRC, viz.: 2% QF-1 (205°C), 13.5 min; 1% XE-60 (207°C) 15.3 min; 2% SE-30 (203°C), 16.9 min; with identical retention times to those of an authentic sample of derivatised GA₂₉, viz.: 2% QF-1 (205°C) 13.5 min; 1% XE-60 (207°C), 15.3 min; 2% SE-30 (203°C), 16.8 min.

2,3 (^3H)-GA₂₉ was stored in 95% ethanol at -20°C .

Application to dwarf pea and extraction. 2,3 (^3H)-GA₂₉ (2.5×10^5 dpm) was applied in 5 μL droplets of 50% ethanol to the plumular hook of 5 day old etiolated pea shoots or dissolved in distilled water and dry, mature seed allowed to imbibe until the radicle had broken through the seed coat. Etiolated shoots were harvested 22 hr. after (^3H)-GA₂₉ application and seeds after 48 hr. Tissues were ground in 80% methanol and extracted as described above under *Preparation of 2,3 (^3H)-GA₂₉*, except that the aqueous phase remaining after ethyl acetate extraction was further partitioned against *n*-butanol at pH 3.0. The acidic ethyl acetate fraction was chromatographed on thin layers of silica gel G using ethyl acetate, chloroform, formic acid (50:50:1 v/v), the gel scraped directly into scintillation vials, eluted with absolute methanol and levels of radioactivity measured in a Packard Tricarb scintillation spectrometer fitted with an absolute activity analyser. Toluene containing 2,4-diphenyl oxazole (5 g/L was used as scintillant).

RESULTS AND DISCUSSION

Gibberellin A₂₀ was converted into GA₂₉ in yields of 30–40 percent by germinating pea seeds. This is an exceptionally high percentage conversion, probably the highest yet recorded for an intact organ of a higher plant. Most previous studies employing radioactive GAs have achieved conversions in the order of 1–5 percent (see Musgrave and Kende, 1970; Durley *et al.*, 1974).

In previous studies (Railton *et al.*, 1974a) it was found that etiolated shoots of dwarf pea converted GA₂₀ into GA₂₉ less efficiently than did germinating seed; conversions of GA₂₀ in shoots being in the order of 3–4 percent. This suggests that marked differences exist between organs of dwarf pea in their ability to metabolise GAs.

The build-up of GA_{29} in seed compared to shoot, could indicate either, a more active $2,\beta$ -hydroxylase enzyme in seed which converts $GA_{20} \rightarrow GA_{29}$, or, a more efficient system in seedlings which metabolises GA_{29} and prevents its

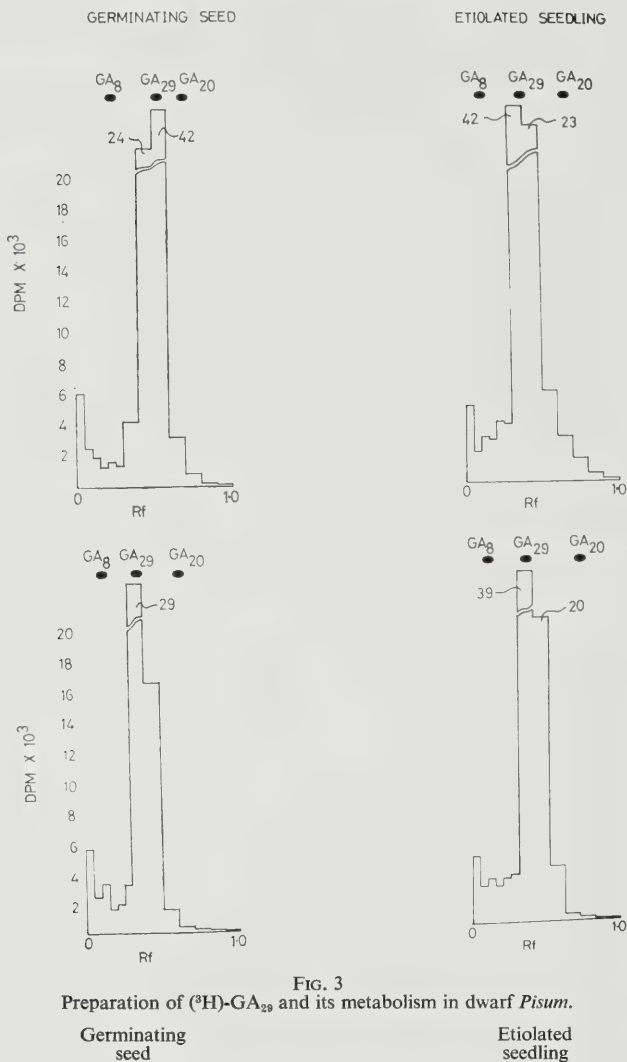


FIG. 3
Preparation of (^3H) - GA_{29} and its metabolism in dwarf *Pisum*.
Germinating seed Etiolated seedling

accumulation. Radioactive GA₂₉ was therefore prepared biosynthetically to help resolve this problem.

2,3 (^3H)-GA₂₉ was converted into a similar spectrum of acidic, radioactive metabolites by both etiolated seedlings and germinating seeds of dwarf *Pisum*. The results of 2 separate experiments are shown in Figure 3. Two main zones of radioactivity were observed; an extremely polar zone (Rf 0.0–0.1) and a less polar zone exhibiting similar chromatographic properties to those of GA₈ (Rf 0.15–0.26). A third zone was also detected which was poorly resolved from GA₂₉ itself, particularly in extracts of seed. GA₂₉ was converted into the most polar zone in yields of 1.5–2.0 percent by germinating seeds and in yields of 0.8–1.0 percent by seedlings. The less polar zone was produced from GA₂₉ in percentage conversions of 0.7–1.0 percent by seeds and 0.4–0.9 percent by seedlings.

The similar chromatographic behaviour of one of the polar metabolites to that of GA₈ suggests that GA₂₉ might be hydroxylated at C-3 to produce this diol. However, it has already been shown in dwarf pea that GA₁, in which C-3 is already hydroxylated, is specifically converted by 2, β -hydroxylation to GA₈ (Railton, Durley, and Pharis, unpublished data). It thus seems unlikely therefore, that GA₈ is indeed produced from GA₂₉. However GA₁ has not, as yet, been detected in dwarf pea (MacMillan, personal communication) and the significance of the conversion of GA₁→GA₈ could be questioned, although the dangers inherent in drawing conclusions from negative data are wellknown. Further, the orientation of tritium atoms at the C-2, C-3 double bond in GA₂₀ is not known and introduction of [OH] at these sites might result in complete displacement of tritium and an inability to detect GA₈. The nature of the very polar metabolite remaining on the origin of chromatograms (Rf 0.0–1.1) is unknown. A similar metabolite zone was detected amongst the conversion products of GA₉ in dwarf pea (Railton *et al.*, 1974) but all attempts to characterise it by GC-MS have so far failed (Railton, Durley and Pharis, unpublished data). The distribution of radioactivity between the diethyl ether, ethyl acetate and *n*-butanol fractions is shown in the Table. Interestingly, the *n*-butanol fractions from both seed and seedlings contained significant levels of radioactivity, suggesting conversion of GA₂₉ and/or its metabolites to very polar compounds such as GA-glycosides. GA₂₉-2-O- β -D-glucopyranosyl ether has been isolated from a higher plant source (Yokota *et al.*, 1970).

In previous work, the highly successful technique of applying "cold carrier" along with the radioactive GA led to the unequivocal characterisation of radioactive GA metabolites in dwarf *Pisum* by GC-MS (Railton, *et al.*, 1974 a, b; Durley *et al.*, 1975; Railton *et al.*, 1975a). In the present work the unavailability of GA₂₉ in reasonable amounts hampered any attempts to increase GA₂₉ metabolite "pool" sizes and so obtain enough mass of product for detailed

spectral analysis. It is obvious that future biosynthetic studies in higher plants involving the use of rarer GAs will be greatly impeded until methods have been devised for the chemical synthesis of these GAs.

TABLE

Distribution of radioactivity between the diethyl ether, acidic ethyl acetate and *n*-butanol fractions from extracts of seed and etiolated seedlings of dwarf *Pisum sativum* treated with (³H)-GA₂₉*.

Expt. No.	SEED			SEEDLING		
	Diethyl ether (dpm)	Ethyl acetate (dpm)	<i>n</i> -butanol (dpm)	Diethyl ether (dpm)	Ethyl acetate (dpm)	<i>n</i> -butanol (dpm)
(1)	0	8,7 x 10 ⁴	1,2 x 10 ³	0	9,7 x 10 ⁴	6,0 x 10 ³
(2)	0	6,6 x 10 ⁴	6,0 x 10 ²	0	8,4 x 10 ⁴	2,2 x 10 ³

*2,5 x 10⁵ dpm per treatment

The present results show that GA₂₉ was metabolised to a similar extent in both germinating seed and etiolated seedlings of dwarf pea. This would suggest therefore that germinating seeds possess a more active 2,β-hydroxylase enzyme system than etiolated shoots rather than a less efficient method for removing accumulating GA₂₉. Recent work in this department has resulted in the isolation of a 2,β-hydroxylase from germinating pea seeds and its properties are currently under investigation.

The problem of differences in percentage conversions of applied radioactive GAs is interesting. The significance for example, of a 1.5–2.0 percent conversion of GA₂₉ into its most polar metabolite in 24 hours, could be questioned as not being particularly meaningful from a biosynthetic point of view. However, GA₂₉ is a native pea GA and it would be expected that the plant would possess the ability to metabolise it efficiently. Apparent low conversion percentage conversions of GAs to their metabolites could be due to two main factors.

- (1) Permeability restrictions allowing only a small percentage of the applied precursor to reach the actual site of interconversion. This could mean that a 2,0 percent conversion based upon the amount of precursor supplied, could represent a 100 percent conversion as far as the plant is concerned.
- (2) Most of the applied precursor reaches the site of interconversion but the plant carefully controls the "pool size" of the metabolites, allowing say, only 2.0 percent of the applied precursor into the "pool" at any time. This suggests that "turnover rates" of GAs and their precursors are subject to fine control mechanisms, a possibility which, on present evidence, cannot be dismissed. In relation to this possibility, time course studies

using the GA precursor *ent*-Kaurenoic acid applied to dwarf rice seedlings showed that the levels of this compound decreased with time and yet were unaccompanied by significant increases in levels of its metabolites (Railton *et al.*, 1975b) suggesting regulation of metabolite "pool" sizes.

This means that quoting a percentage conversion for an applied radioactive GA only indicates the amount of product in any "pool" at any one time and is therefore not a true indication of how much GA has actually been metabolised. More satisfactory data could be achieved therefore, if the "turnover" rates of individual GAs could be determined. Preliminary attempts have been made to investigate "turnover" rates of GA₂₀ in dwarf *Pisum* (Railton, 1974 a, b) and, although not fully satisfactory at the present time, they offer a method of obtaining more meaningful information regarding the metabolism of labelled GAs in higher plants.

The present results indicate that germinating pea seeds metabolise different GAs in a biosynthetic sequence at different rates and mechanisms must therefore exist to control these rates of metabolism. The rapid accumulation of GA₂₉, a GA exhibiting low biological activity, suggests that pea seeds possess a mechanism for the efficient, deactivation of biologically active, GA₂₀. The significance of this remains to be determined.

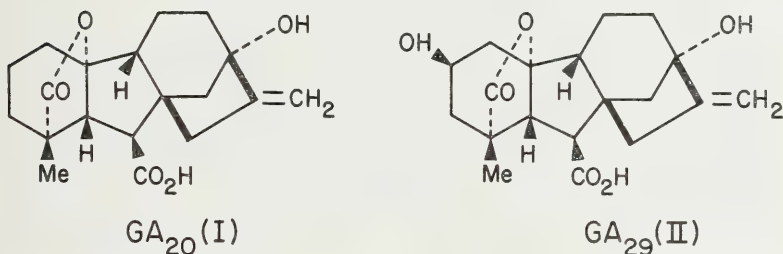


FIG. 4
Structures of GA₂₀ and GA₂₉. Preparation of (^3H)-GA₂₉.

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